

# The Effect of Photochemotherapy (PUVA) on Cell Mediated Immunity in the Guinea Pig

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Guinea pigs were injected intraperitoneally with 8-methoxypsoralen (8-MOP) and afterwards the shaved right flank was irradiated with UV-A. This treatment was performed daily for 14 days. Seven days after treatment started they were immunized with ovalbumin in Freund's complete adjuvant and 7 days later skin tested with ovalbumin on the unirradiated left flank. Photochemotherapy (8-MOP plus UV-A) did not alter the total white blood cells or the proportion of lymphocytes or granulocytes. However, this treatment significantly depressed the delayed hypersensitivity reaction to ovalbumin; nonspecific inflammation, induced by intradermal turpentine, was not altered. In addition, treatment with 8-MOP alone suppressed the skin reaction to ovalbumin, but not to the same extent as treatment with 8-MOP and UV-A. 8-MOP plus UV-A had no effect on macrophage migration inhibition factor but it did significantly depress mitogen and antigen specific lymphocyte transformation.

Photochemotherapy with oral 8-methoxypsoralen (8-MOP) and whole-body long wavelength ultraviolet radiation (UV-A: 320 to 400 nm; maximum at 365 nm) is becoming increasingly popular in the treatment of psoriasis [1,2], mycosis fungoides [3], and other skin disorders. Abnormal cellular proliferation plays an important role in the pathogenesis of some of these diseases. The mode of action of photochemotherapy is therefore believed to be mainly related to the ability of 8-MOP to inhibit cellular replication by forming mono-adducts and cross-links with DNA in the presence of UV-A [4,5]. Some of the long-term side effects of photochemotherapy, including its potential to initiate skin cancer, may also be related to this property.

It is thought that immunological reactions play some part in both the etiology of most diseases which respond to photochemotherapy, and in the side effects produced by this treatment. The experimental evidence presently available suggests that the immunological effects of photochemotherapy could be due to a direct effect on 8-MOP sensitized, immunological-competent cells circulating within each of the UV-A in the superficial dermis [6]. If this is so, the result would be an effect on systemic immune function, and this could be monitored by appropriate skin tests performed at a distant nonirradiated site in sensitized individuals. It would of course be important to determine the effects of photochemotherapy on systemic immune function in

the healthy organism before investigating its effects in disease states.

In this project we have investigated the effect of 8-MOP and UV-A (PUVA) treatment on the cell-mediated immune response in the guinea pig. PUVA treatment was found to depress the delayed hypersensitivity reactions to ovalbumin (OA) in animals immunized with OA in Freund's complete adjuvant (FCA). Photochemotherapy had no effect on the *in vitro* parameter of cell mediated immunity—macrophage migration inhibition factor, although it did significantly depress mitogen and antigen specific lymphocyte transformation.

## MATERIALS AND METHODS

### Animals

Female outbred Hartley strain guinea pigs, weighing 300–350 gm were used. They were purchased from Porcellus Animal Breeding Ltd., Firgrove Farm, Heathfield, Sussex, and fed on pelleted diet RGP (F. Dixon & Son, Ware, Herts) supplemented with hay and ascorbic acid (10 mg/day/animal). The animals were housed in a room with subdued lighting well away from direct sunlight.

### Antigens

Ovalbumin (OA), crystallized 5 times, was purchased from Miles Serevac Ltd., Maidenhead, Berks. Phytohemagglutinin (PHA-Reagent grade) was purchased from Wellcome Reagents Ltd.

### Immunization Procedures

OA was dissolved in physiological saline, emulsified with an equal volume of Freund's complete adjuvant (FCA, Difco containing *Mycobacterium butyricum*) and 0.1 ml injected into each footpad. The animals received a total of 10 µg OA.

### Skin Tests

Guinea pigs were skin tested on the left flank 7 days after immunization (day 14 of the experiment) and reactions were read 24 and 48 hr later. Animals immunized with OA were skin tested by intradermal injection of 100 µg OA dissolved in 0.1 ml saline.

Reactions were assessed by measuring the increase in skin fold thickness using skin callipers (Schelltäster, Kröplin AO2T). The results are expressed as increase in skin fold thickness which represents the reading at the skin test site minus the average thickness of the normal skin on both sides of the reaction. Animals immunized for macrophage migration inhibition and lymphocyte transformation were not skin tested, as a skin test at 7 days suppresses these results.\*

Unimmunized guinea pigs were skin tested by an intradermal injection of 0.1 ml turpentine diluted in olive oil to a concentration of 1:10 to measure nonspecific skin reactivity. The inflammatory reactions produced were assessed as for the OA skin reaction.

### PUVA Treatment

Guinea pigs were injected intraperitoneally with 8-methoxypsoralen (8-MOP, Promedica, France) 45 min before exposure to UV-A. The animals received 0.75 mg 8-MOP in 0.1 ml of vehicle made up of acetone:propylene glycol:ethyl alcohol (10:10:80). The UV-A light source (PUVA '4000'-Herbert Waldmann, Germany) was fitted with Sylvania Lifeline/FR90T12/PUVA/HO tubes, and an inbuilt photoelec-

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#### Abbreviations:

- FCA: Freund's complete adjuvant
- LTT: Lymphocyte transformation test
- MI: migration inhibition index
- MIF: macrophage migration
- 8-MOP: 8-methoxypsoralen
- OA: ovalbumin
- PEC: peritoneal exudate cells
- S1: stimulation index

tric UV-A dosimeter. This source emits a continuous spectrum of irradiation between 320–400 nm wavelength with peak emission at 365 nm. The guinea pigs received a UV-A skin surface dose of 2 joules/cm<sup>2</sup>, which was given to a shaved area of skin, approximately 9 × 5 cm on the right flank, which was depilated every third day. The animals were held singly in wire cages during exposure.

### Histology

Skin reaction sites were excised 24 hr after skin test, fixed in Bouin's solution, sectioned at 5 µm and stained with hematoxylin eosin. The intensity of the infiltrate was assessed on an arbitrary scale of 3, 2, 1, 0, where 3 indicates an intense cellular infiltrate, 2 moderate infiltration, 1 scanty infiltration and for 0 there is no cellular infiltrate. In Table III the results were expressed as mean ± standard deviation in a minimum of 6 animals.

### Red Counts

Approximately 0.5 ml of blood was bled from the guinea pig ear on days 0, 7 and 14, into heparinized tubes. Total differential white cell counts were made.

### Inhibition of Macrophage Migration (MIF)

Oil induced peritoneal exudate cells (PEC) were obtained from immunized guinea pigs. The PEC were washed twice in Eagles MEM and resuspended to a final concentration of  $4.5 \times 10^7$  viable cells/ml. Viability was assessed by trypan blue exclusion. Migration inhibition, using the capillary tube method, was assayed against 1000, 100, and 10 µg/ml OA. Four capillary tubes were set up against each antigen concentration. The mitogen areas were projected, traced out and measured with a planimeter. The results are expressed as migration inhibition index (MI) calculated using the formula:

$$\text{MI} = \frac{\text{Area of migration with antigen}}{\text{Area of migration without antigen}}$$

### Lymphocyte Transformation Test (LTT)

Blood lymphocytes were separated by centrifugation on Ficoll-Hypaque (12.5 g Hypaque, 6.35 g Ficoll in 100 ml distilled water). The separated cells were washed twice with Eagles MEM and resuspended in RPMI 1640 (Gibco) to a concentration of  $1.2 \times 10^6$  viable cells/ml. RPMI with 2 mM L-glutamine,  $10^4$  units penicillin/ml, 10 mg streptomycin/ml and 10% guinea pig serum was used throughout.

200 µl of cell suspension ( $2.4 \times 10^5$  viable cells) were added to round bottomed wells of microtitre culture plates (Nuclea-Denmark). 20 µl of antigen or mitogen were added to groups of 4 wells. As a control, cells in 4 wells were cultured without antigen. The concentrations of OA used were 10, 100, and 1000 µg/ml and that of PHA was 9.3 µg/ml sterile saline. The cultures were kept for 72 hr in a humidified incubator at 37°C, in an atmosphere of 5% CO<sub>2</sub>-95% air and pulsed with 0.75 µCi <sup>3</sup>H-thymidine (23.26 Ci/mmol, The Radiochemical Centre, Amersham, Bucks). Five hours later the cells were harvested with a Mash II harvester (Microbiologic Associated Inc) onto Whatman grade CF/A paper. The radioactivity on the filter discs was counted in a Packard Tricarb Scintillation Counter (Model 2405). The results are expressed as Stimulation Index (SI) where

$$\text{SI} = \frac{\text{Mean } ^3\text{H-thymidine uptake of cells cultured with antigen}}{\text{Mean } ^3\text{H-thymidine uptake of cells cultured without antigen}}$$

### Statistics

P-values were determined using the Student's *t*-test.

### Experimental Design

Animals received either 8-MOP plus UV-A, UV-A alone or 8-MOP alone daily for a total of 14 days. This exposure to UV-A did not produce erythema on the shaved flanks of any of the groups of guinea pigs subjected to irradiation. Seven days after the start of treatment (day 7) the guinea pigs were immunized and 7 days later, the day after treatment was finished (day 14), the animals were skin tested. A control group with no treatment was set up in parallel with each experiment. Other controls were one group that received intraperitoneal injection of the 8-MOP vehicle alone and another group received intraperitoneal injections of physiological saline. The *in vitro* tests (MIF and LTT) were done on day 21. Each group contained a minimum of 6 guinea pigs.

## RESULTS

### Blood Counts

Five groups of guinea pigs were set up and blood taken from them on day 0 before treatment began, day 7 before immunization and day 14 before skin testing. As shown in Table I, none of the treatments had any effect on either the total white blood cells or the lymphocytes or granulocytes. The rise in all the mean cell counts on day 14, although not significant, is probably due to immunization with OA in FCA, on day 7.

### Delayed Hypersensitivity Skin Reactions

As shown in Table II, PUVA treatment and 8-MOP alone significantly suppressed the delayed hypersensitivity skin reactions at 24 hr. In addition, PUVA treatment suppressed the skin reaction as compared to 8-MOP treatment ( $p < 0.02$ ) and the skin reactions in the PUVA-treated animals were still suppressed at 48 hr. The skin reactions were unaltered by treatment with UV-A alone, the 8-MOP vehicle or saline. None of the treatments altered the nonspecific inflammation induced in unimmunized guinea pigs by the intradermal injection of turpentine.

### Histology

An assessment was made of the cellular infiltrate round the blood vessels superficial to the panniculus carnosus and immediately under the epidermis in skin sites excised 24 hr after skin test. PUVA treatment caused a marked decrease in the infiltrate round the blood vessels over the panniculus carnosus (Table III). The decrease in the subepidermal infiltrate was not so great. 8-MOP and UV-A caused a lesser decrease in the perivascular infiltrate and this was mainly in polymorphonu-

TABLE I. The effect of photochemotherapy on the peripheral white blood cells

| Treatment         | Time               | Total        | Lymphocytes | Granulocytes |
|-------------------|--------------------|--------------|-------------|--------------|
| —                 | Day 0 <sup>a</sup> | 10127 ± 2680 | 4448 ± 1479 | 5682 ± 2387  |
| —                 | Day 7              | 12083 ± 3364 | 5769 ± 1344 | 6314 ± 2636  |
| PUVA <sup>b</sup> |                    | 8650 ± 1679  | 4206 ± 967  | 4446 ± 1383  |
| 8-MOP             |                    | 9450 ± 3270  | 4246 ± 1435 | 5203 ± 2440  |
| UV-A              |                    | 9983 ± 2163  | 4961 ± 2436 | 5023 ± 1201  |
| Vehicle           |                    | 12633 ± 2716 | 4891 ± 1365 | 7751 ± 2343  |
| —                 | Day 14             | 11216 ± 5141 | 4168 ± 2289 | 7049 ± 3023  |
| PUVA              |                    | 14650 ± 5421 | 3695 ± 777  | 10955 ± 5431 |
| 8-MOP             |                    | 15667 ± 5203 | 4706 ± 1359 | 10960 ± 4009 |
| UV-A              |                    | 18500 ± 5320 | 5703 ± 705  | 12797 ± 5382 |
| Vehicle           |                    | 15467 ± 4836 | 6232 ± 1428 | 9234 ± 4209  |

<sup>a</sup> Blood was collected on Day 0, before the experiment started, day 7 before immunization and day 14 before skin test.

<sup>b</sup> Treatments made daily for 14 days with 0.75 mg. 8-MOP administered 45 min prior to UV-A exposure.

TABLE II. Effect of photochemotherapy on delayed hypersensitivity skin reactions in guinea pigs immunized with ovalbumin in Freund's complete adjuvant

| Treatment         | No. of animals | Increase in skin thickness (10 <sup>-1</sup> mm) ± SD |                          |
|-------------------|----------------|---|--------------------------|
|                   |                | 24 hr   | 48 hr                    |
| —                 | 24             | 11.4 ± 3.9  | 8.9 ± 4.1                |
| PUVA <sup>d</sup> | 22             | 5.4 ± 3.4 <sup>a,c</sup>                              | 3.7 ± 1.8 <sup>b,c</sup> |
| 8-MOP             | 24             | 7.9 ± 2.9 <sup>a</sup>                                | 5.4 ± 1.9                |
| UV-A              | 18             | 11.4 ± 4.2  | 10.1 ± 3.3               |
| Vehicle           | 6              | 13.0 ± 4.4  | 9.2 ± 2.6                |
| Saline            | 6              | 12.9 ± 3.9  | 10.2 ± 3.1               |

<sup>a</sup>  $p < 0.001$  as compared to untreated.

<sup>b</sup>  $p < 0.02$  as compared to untreated.

<sup>c</sup>  $p < 0.02$  as compared to 8-MOP treated.

<sup>d</sup> Treatments made daily for 14 days with 0.75 mg. 8-MOP administered 45 min prior to UV-A exposure.

TABLE III. Effect of PUVA, 8-MOP or UV-A on the cellular infiltrate in dermis of guinea pigs sensitized with ovalbumin (OA) in Freund's complete adjuvant and skin tested with OA 24 hr previously<sup>a</sup>

|                            | Control   | PUVA                    | 8-MOP                  | UV-A                   |
|----------------------------|-----------|-------------------------|------------------------|------------------------|
| A. Perivascular infiltrate | 2.9 ± 0.4 | 1.0 ± 0.5 <sup>b</sup>  | 1.7 ± 0.5 <sup>b</sup> | 1.8 ± 0.4 <sup>b</sup> |
| Polymorphonuclear          | 1.9 ± 0.4 | 0.7 ± 0.3 <sup>b</sup>  | 0.8 ± 0.3 <sup>b</sup> | 0.8 ± 0.3 <sup>b</sup> |
| Mononuclear                | 2.6 ± 0.8 | 1.1 ± 0.5 <sup>c</sup>  | 1.8 ± 0.4              | 1.8 ± 0.4              |
| B. Subepidermal infiltrate | 2.6 ± 0.5 | 1.50 ± 0.5 <sup>c</sup> | 1.7 ± 0.5              | 2.5 ± 0.5              |
| Polymorphonuclear          | 1.0 ± 0   | 0.8 ± 0.2               | 0.9 ± 0.6              | 1.2 ± 0.4              |
| Mononuclear                | 2.3 ± 0.5 | 1.7 ± 0.5               | 1.7 ± 0.5              | 2.5 ± 0.5              |

<sup>a</sup> Treatments made daily for 14 days with 0.75 mg. 8-MOP administered 45 min prior to UV-A exposure.

<sup>b</sup>  $p < 0.001$  as compared to controls.

<sup>c</sup>  $p < 0.01$  as compared to controls.

Note: Intensity of infiltrate assessed on a scale of 3,2,1,0. Results expressed as mean ± standard deviation of a minimum of 6 animals.

clear leukocytes, whereas the effect of PUVA was on both polymorphonuclear cells and mononuclear cells.

### Inhibition of Macrophage Migration

Peritoneal exudate cells were collected from guinea pigs 14 days after immunization and inhibition of macrophage migration assessed in the presence of OA. These animals were not skin tested on day 7 as this depresses macrophage migration. None of the treatments had any effect on the macrophage migration: in all instances the migration index, in the presence of varying concentrations of OA, was the same as the untreated animals (Table IV).

### Lymphocyte Transformation

Lymphocyte transformation to PHA and OA was assayed on blood lymphocytes collected 14 days after immunization. Again the guinea pigs were not skin tested as this affects the lymphocyte transformation. As the control counts, i.e., the counts/min in the cultures without antigen or mitogen, were unaffected by any of the treatments the stimulation indices only are reported (Table V). As can be seen, total PUVA treatment significantly depressed the stimulation index to PHA and to all concentrations of OA as compared with the control group. 8-MOP or UV-A alone did not significantly depress the stimulation indices to either PHA or OA.

## DISCUSSION

In a previous study a depressed ability to mount a contact hypersensitivity reaction to dinitrochlorobenzene has been reported in psoriatic patients who have been exposed to total body PUVA [7]. As the site of contact reaction on the uninvolved psoriatic skin had been exposed to UV-A, the depressed reactions could have been due to nonspecific changes in the skin.

Using the guinea pig model it has been possible to examine the effect of PUVA therapy on normal skin. By exposing one flank to the UV light and skin testing on the other flank, the skin test could be performed on normal, nonirradiated skin. A significant suppression of the tuberculin type of delayed hypersensitivity reaction was observed in animals immunized with OA in FCA and receiving PUVA therapy. These results confirm those reported by Morrison, Woehler, and Parrish [8] who found depressed delayed hypersensitivity reactions to dinitrophenylated bovine globulin after guinea pigs had been exposed to 8-MOP plus UV-A. In addition, we found that injection of 8-MOP alone caused significant suppression of the skin reaction. Although this was not as great as that caused by PUVA therapy, it was found in all 4 separate experiments and was biologically significant. As none of the treatments had any effect on non-specific inflammation induced by turpentine, it is clear that photochemotherapy specifically affects the immunological system. Further evidence for this was found when the skin sites were examined histologically. PUVA treatment caused a significant decrease in both mononuclear cells and polymorphonuclear leukocytes, whereas 8-MOP and UVA only affected the

TABLE IV. Effect of chemotherapy on macrophage migration index of animals immunized with ovalbumin in Freund's complete adjuvant

| Treatment         | Migration Index<br>OA concentration (µg/ml) |             |             |
|-------------------|---|-------------|-------------|
|                   | 1000  | 100         | 10          |
| —                 | 0.54 ± 0.04                                 | 0.59 ± 0.09 | 0.62 ± 0.10 |
| PUVA <sup>a</sup> | 0.57 ± 0.06                                 | 0.58 ± 0.12 | 0.61 ± 0.12 |
| 8-MOP             | 0.60 ± 0.05                                 | 0.67 ± 0.05 | 0.69 ± 0.06 |
| UV-A <sup>c</sup> | 0.55 ± 0.05                                 | 0.58 ± 0.06 | 0.69 ± 0.06 |

<sup>a</sup> Treatments made daily for 14 days with 0.75 mg 8-MOP administered 45 min prior to UV-A exposure.

TABLE V. Effect of photochemotherapy on lymphocyte transformation to PHA and OA in animals immunized with ovalbumin (OA) in Freund's complete adjuvant

| Treatment         | PHA                      | Stimulation Index ± SD<br>OA (µg/ml) |                          |                          |
|-------------------|--------------------------|--------------------------------------|--------------------------|--------------------------|
|                   |                          | 1000                                 | 100                      | 10                       |
| —                 | 4.54 ± 1.27              | 2.45 ± 1.20                          | 3.01 ± 1.39              | 2.56 ± 1.42              |
| PUVA <sup>c</sup> | 2.32 ± 0.65 <sup>a</sup> | 1.23 ± 0.27 <sup>b</sup>             | 1.15 ± 0.26 <sup>a</sup> | 1.35 ± 0.30 <sup>a</sup> |
| 8-MOP             | 5.54 ± 2.84              | 1.66 ± 1.19                          | 2.14 ± 1.47              | 2.10 ± 1.13              |
| UV-A              | 5.60 ± 2.48              | 2.83 ± 1.90                          | 2.89 ± 1.00              | 2.52 ± 0.54              |

<sup>a</sup>  $0.01 > p > 0.001$  as compared to controls.

<sup>b</sup>  $0.05 > p > 0.01$  as compared to controls.

<sup>c</sup> Treatments made daily for 14 days with 0.75 mg. 8-MOP administered 45 min. prior to UV-A exposure.

polymorphonuclear cells.

There is conflicting evidence in the literature concerning the *in vivo* effect of PUVA therapy on peripheral lymphocytes from psoriatic patients. Morrison et al [6] studied the response to PHA of lymphocytes from 11 psoriatic patients under PUVA therapy. They reported that the responsiveness tended to fall during the first week of treatment and then rose towards pretreatment levels. Friedmann and Rogers [9] studying similar psoriatic patients under PUVA therapy showed that lymphocytes removed immediately after UV-A irradiation showed less <sup>3</sup>H-thymidine incorporation than cells taken just prior to irradiation. In contrast Fraki, Eskela, and Hopsu-Havu [10] did not find any change in the mitogen response of peripheral blood lymphocytes of psoriatic patients after 12 weeks of PUVA therapy. Similar results have been reported by other workers [11]. Differences in the UV-A irradiation schedules, the dose of 8-MOP, the time of collection of blood after PUVA treatment and variability in the *in vitro* technology may account for the discrepancy in the above findings.

Our *in vitro* findings, on healthy animals rather than diseased humans, would agree with those of Friedmann and Rogers [9] and Morrison et al [6]. A significant decrease in the response of lymphocytes to PHA was found in animals that had received PUVA treatment. In addition, we demonstrate a significant decrease in antigen specific lymphocyte transformation. UV-A or 8-MOP alone did not affect the <sup>3</sup>H-thymidine uptake in the



presence of either PHA or OA. The effect of PUVA on lymphocyte transformation may be cumulative as the lymphocyte transformation test was carried out 7 days after the last PUVA treatment. In contrast to the lymphocyte transformation results, macrophage migration inhibition was not affected by PUVA. In this instance this may be because the assay was carried out 7 days after the cessation of photochemotherapy. However, it has been previously reported that exposure to fluorescent light prevented lymphocytes from proliferating in the presence of antigen, but did not affect the production of MIF [12]. It would seem that PUVA treatment suppresses those lymphocytes responsible for LTT but does not affect the smaller number of lymphocytes capable of producing MIF.

It is difficult to understand why the treatment with 8-MOP alone should depress the delayed hypersensitivity reactions to OA. As the animals were housed in an unlit room, which could not have received any direct sunlight, it is not possible that they were exposed to UV-A from other sources. Although the dose of 8-MOP per kilo body weight was 4 times the therapeutic dose, there is no evidence that this was toxic as the LTT and MIF assays were not suppressed by 8-MOP. This, despite the fact that it has been reported that lymphocyte proliferation can be suppressed by *in vitro* treatment with 8-MOP in the dark [13].

As 8-MOP, in the dark, has been reported to produce mutations in *Escherichia coli*, presumably due to an effect on the DNA [14], it is possible that it produces a similar effect on the effector lymphocytes responsible for the skin reaction in cell mediated immunity. As reported here, PUVA treatment has a preferential effect on the lymphocytes responsible for LTT rather than those responsible for MIF production, it may be that a similar preferential effect is found with 8-MOP as regards the skin reaction. Alternatively, it could be that 8-MOP affects the cells responsible for the presentation of antigen. For instance, it has been reported, in mice, that UV light of a different wavelength (280–340 nm) causes a defect in the spleen and peritoneal exudate adherent cells responsible for the presentation of antigen [15].

Although this study has shown that PUVA therapy suppresses the delayed hypersensitivity skin reaction it is not yet clear whether the treatment affects the skin test by altering lymphocytes as they pass through the skin or whether the effect is systemic, preventing the animal from mounting a cell mediated immune reaction. From the histological study it is evident that the PUVA therapy has had some effect on the cells in the skin, even though the skin test site was not directly exposed to irradiation. The lymphocyte transformation results also indicate that PUVA has a systemic effect. Thus it would

appear that in normal healthy animals, PUVA therapy probably suppresses at least 2 parameters of a cell-mediated immune reaction. As PUVA therapy has a direct effect on generalized lymphocyte function, care should be taken when assessing the need for this form of the therapy in the future. The implications of these findings need to be investigated further.

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